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(71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street – R440, Emeryville, CA 94608 (US).

(72) Inventors: GRANOFF, Dan, M.; Chiron Corporation, 4560 Horton Street – R440, Emeryville, CA 94608 (US). GREER, Catherine, E.; Chiron Corporation, 4560 Horton Street – R440, Emeryville, CA 94608 (US). VAN NEST, Gary, A.; Chiron Corporation, 4560 Horton Street – R440, Emeryville, CA 94608 (US).

(74) Agents: HARBIN, Alisa, A.; Chiron Corporation, Intellectual Property – R440, P.O. Box 8097, Emeryville, CA 94662–8097 (US) et al.

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(54) Title: USE OF VIRUS–LIKE PARTICLES AS ADJUVANTS

(57) Abstract

Adjuvant formulations are provided that contain nonreplicating virus–like particles (VLPs). The VLPs can be derived from any of several viruses and are useful for enhancing the immunological response to a selected antigen, wherein the antigen is distinct from the particles.

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USE OF VIRUS-LIKE PARTICLES AS ADJUVANTS

Technical Field

The present invention relates generally to agents which enhance the immune response to a selected antigen. In particular, the invention pertains to the use of virus-like particles (VLPs) as adjuvants and coadjuvants, formulations including the VLPs, and methods for making and using the compositions of the invention.

Background

Vaccine compositions often include immunological adjuvants to enhance cell-mediated and humoral immune responses. For example, depot adjuvants are frequently used which adsorb and/or precipitate administered antigens and which can retain the antigen at the injection site. Typical depot adjuvants include aluminum compounds and water-in-oil emulsions. However, depot adjuvants, although increasing antigenicity, often provoke severe persistent local reactions, such as granulomas, abscesses and scarring, when injected subcutaneously or intramuscularly. Other adjuvants, such as lipopolysacharrides and muramyl dipeptides, can elicit pyrogenic responses upon injection and/or Reiter's symptoms (influenza-like symptoms, generalized joint discomfort and sometimes anterior uveitis, arthritis and urethritis).

Particulate carriers with adsorbed or entrapped antigens have been used in an attempt to circumvent these problems. Such carriers present

multiple copies of a selected antigen to the immune system and promote trapping and retention of antigens in local lymph nodes. The particles can be phagocytosed by macrophages and can enhance antigen presentation through cytokine release. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG. While offering significant advantages over other more toxic systems, antigen-containing PLG microparticles suffer from some drawbacks. For example, the production of microparticles is difficult and involves the use of harsh chemicals that can denature the antigen and destroy the immunogenicity thereof. Furthermore, antigen instability can occur due to the high shear forces used to prepare small microparticles and due to interfacial effects within the emulsions used.

Liposomes have also been employed in an effort to overcome these problems. Liposomes are microscopic vesicles formed from lipid constituents such as phospholipids which are used to entrap pharmaceutical agents. Although the use of liposomes as a drug delivery system alleviates some of the problems described above, liposomes exhibit poor stability during storage and use, and large scale production and manufacturing of liposomes is problematic.

Viral particles can be used as a matrix for the proper presentation of an antigen entrapped or associated therewith to the immune system of the host. For example, U.S. Patent No. 4,722,840 describes hybrid particles comprised of a particle-forming fragment of a structural protein from a virus, such as a particle-forming fragment of hepatitis B virus (HBV) surface antigen (HBsAg), fused to a heterologous

polypeptide. Tindle et al., *Virology* (1994) 200:547-557, describes the production and use of chimeric HBV core antigen particles containing epitopes of human papillomavirus (HPV) type 16 E7 transforming protein.

Adams et al., *Nature* (1987) 329:68-70, describes the recombinant production of hybrid HIVgp120:Ty VLPs in yeast and Brown et al., *Virology* (1994) 198:477-488, the production of chimeric proteins consisting of the VP2 protein of human parvovirus B19 and epitopes from human herpes simplex virus type 1, as well as mouse hepatitis virus A59. Wagner et al., *Virology* (1994) 200:162-175, Brand et al., *J. Virol. Meth.* (1995) 51:153-168 and Wagner et al., *Virology* (1996) 220:128-140, describe the assembly of chimeric HIV-1 p55*gag* particles. U.S. Patent No. 5,503,833 describes the use of rotavirus VP6 spheres for encapsulating and delivering therapeutic agents.

Despite the above antigen-presentation systems, there is a continued need for effective and safe adjuvants for use in a variety of pharmaceutical compositions and vaccines.

Summary of the Invention

The inventors herein have found, surprisingly, that combining selected antigens with VLPs, wherein the antigens are distinct from the VLP, provides for enhanced immune responses to the antigen in question.

In one embodiment, then, the subject invention is directed to a vaccine composition comprising a VLP adjuvant, a selected antigen and a pharmaceutically acceptable excipient. The selected antigen is distinct from the VLP. In particularly preferred embodiments, the VLP is derived from one or more particle-forming polypeptides of a hepatitis

surface antigen or from a particle-forming polypeptide of papillomavirus L1 and/or L2 protein and the selected antigen is a MenC/Hib oligosaccharide conjugate or an HPV E7.

In another embodiment, the subject invention is directed to a method for producing an enhanced immune response in a vertebrate subject comprising administering to the vertebrate subject a VLP adjuvant and a selected antigen. The antigen is distinct from the VLP and the VLP is administered in an amount effective for eliciting an enhanced immune response to the antigen in the vertebrate subject. The VLP can be administered to the subject prior or subsequent to, or concurrent with, the antigen.

In yet another embodiment, the invention is directed to a method for preparing an adjuvant formulation comprising providing a VLP and combining the VLP with a pharmaceutically acceptable excipient.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 depicts antibody responses of infant baboons to vaccines including hepatitis B virus VLPs (HBV). The results are presented as the geometric mean of antibody concentrations in IU/ml.

Figure 2 shows antibody responses of infant baboons to vaccines including HBV VLPs. The results are presented as the geometric mean of antibody concentrations in IU/ml.

Figure 3 shows anticapsular antibody responses of infant baboons to vaccines including HBV VLPs. The results are presented as the geometric mean of antibody concentrations in IU/ml.

Figure 4 depicts anticapsular antibody responses of infant baboons to vaccines including HBV VLPs. The results are presented as the geometric mean of antibody concentrations in IU/ml.

Figures 5A and 5B show serum antibody responses of infant baboons to vaccines including HBV VLPs. Figure 5A shows the IgG anticapsular antibody responses determined by ELISA. Figure 5B depicts complement-mediated bactericidal antibody responses.

Figure 6 is a graph showing the effect of a combined HPV6b L1 VLP and E7 vaccine on rabbit humoral immune responses.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of virology, chemistry, biochemistry, recombinant technology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., *Virology*, 3rd Edition, vol. I & II (B.N. Fields and D.M. Knipe, eds., 1996); *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); *Methods In Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.); *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell, eds., 1986, Blackwell Scientific Publications); Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); and *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.).

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

As used herein, the term "virus-like particle" or "VLP" refers to a nonreplicating, empty viral shell, derived from any of several viruses discussed further below. VLPs are generally composed of one or more viral proteins, such as, but not limited to those proteins referred to as capsid, coat, shell, surface and/or envelope proteins, or particle-forming polypeptides derived from these proteins. VLPs can form spontaneously upon recombinant expression of the protein in an appropriate expression system. Methods for producing particular VLPs are known in the art and discussed more fully below. The presence of VLPs following recombinant expression of viral proteins can be detected using conventional techniques known in the art, such as by electron microscopy, X-ray crystallography, and the like. See, e.g., Baker et al., *Biophys. J.* (1991) 60:1445-1456; Hagensee et al., *J. Virol.* (1994) 68:4503-4505. For example, cryoelectron microscopy can be performed on vitrified aqueous samples of the VLP preparation in question, and images recorded under appropriate exposure conditions.

By "particle-forming polypeptide" derived from a particular viral protein is meant a full-length or near full-length viral protein, as well as a fragment thereof, or a viral protein with internal deletions, which has the ability to form VLPs under conditions that favor VLP formation. Accordingly, the polypeptide may comprise the full-length sequence, fragments, truncated and partial sequences, as well as analogs and precursor forms of the reference molecule. The term therefore intends deletions, additions and

substitutions to the sequence, so long as the polypeptide retains the ability to form a VLP. Thus, the term includes natural variations of the specified polypeptide since variations in coat proteins often occur between viral isolates. The term also includes deletions, additions and substitutions that do not naturally occur in the reference protein, so long as the protein retains the ability to form a VLP.

Preferred substitutions are those which are conservative in nature, i.e., those substitutions that take place within a family of amino acids that are related in their side chains. Specifically, amino acids are generally divided into four families: (1) acidic -- aspartate and glutamate; (2) basic -- lysine, arginine, histidine; (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar -- glycine, asparagine, glutamine, cystine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. For example, it is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. Proteins having substantially the same amino acid sequence as the reference molecule, but possessing minor amino acid substitutions that do not substantially affect the immunogenicity of the protein, are therefore within the definition of the reference polypeptide.

A VLP is "distinct from" from a selected antigen when the antigen is not entrapped within the VLP and/or the antigen and VLP are not expressed together as a fusion protein. However, a VLP is

considered "distinct from" a selected antigen even if there is a loose physical association between the antigen and VLP.

An "antigen" refers to a molecule containing one or more epitopes (either linear, conformational or both) that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is used interchangeably with the term "immunogen." Normally, a B-cell epitope will include at least about 5 amino acids but can be as small as 3-4 amino acids. A T-cell epitope, such as a CTL epitope, will include at least about 7-9 amino acids, and a helper T-cell epitope at least about 12-20 amino acids. The term "antigen" denotes both subunit antigens, i.e., antigens which are separate and discrete from a whole organism with which the antigen is associated in nature, as well as killed, attenuated or inactivated bacteria, viruses, fungi, parasites or other microbes. Antibodies such as anti-idiotype antibodies, or fragments thereof, and synthetic peptide mimotopes, which can mimic an antigen or antigenic determinant, are also captured under the definition of antigen as used herein. Similarly, an oligonucleotide or polynucleotide which expresses an antigen or antigenic determinant *in vivo*, such as in gene therapy and DNA immunization applications, is also included in the definition of antigen herein.

For purposes of the present invention, antigens can be derived from any of several known viruses, bacteria, parasites and fungi, as described more fully below. The term also intends any of the various tumor antigens. Furthermore, for purposes of the present invention, an "antigen" refers to a protein which includes modifications, such as deletions, additions and substitutions (generally

conservative in nature), to the native sequence, so long as the protein maintains the ability to elicit an immunological response, as defined herein. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the antigens.

An "immunological response" to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to the antigen present in the composition of interest. For purposes of the present invention, a "humoral immune response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells ("CTL"s). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the intracellular destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells.

A composition or vaccine that elicits a cellular immune response may serve to sensitize a vertebrate subject by the presentation of antigen in

association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-specific T-lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T-lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, e.g., Erickson et al., *J. Immunol.* (1993) 151:4189-4199; Doe et al., *Eur. J. Immunol.* (1994) 24:2369-2376.

Thus, an immunological response as used herein may be one which stimulates the production of CTLs, and/or the production or activation of helper T-cells. The antigen of interest may also elicit an antibody-mediated immune response. Hence, an immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor T-cells and/or $\gamma\delta$ T-cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, well known in the art.

A vaccine composition which contains a selected antigen along with a VLP adjuvant of the present invention, or a vaccine composition which is coadministered with the subject VLP adjuvant, displays

"enhanced immunogenicity" when it possesses a greater capacity to elicit an immune response than the immune response elicited by an equivalent amount of the antigen administered without the VLP adjuvant or coadjuvant. Thus, a vaccine composition may display "enhanced immunogenicity" because the antigen is more strongly immunogenic or because a lower dose or fewer doses of antigen are necessary to achieve an immune response in the subject to which the antigen is administered. Such enhanced immunogenicity can be determined by administering the VLP composition and antigen controls to animals and comparing antibody titers and/or cellular-mediated immunity against the two using standard assays such as radioimmunoassay and ELISAs, well known in the art.

For purposes of the present invention, an "effective amount" of a VLP adjuvant will be that amount which enhances an immunological response to a coadministered antigen.

By "vertebrate subject" is meant any member of the subphylum chordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The system described above is intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

By "pharmaceutically acceptable" or

"pharmacologically acceptable" is meant a material which is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the VLP adjuvant formulation without causing any undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

By "physiological pH" or a "pH in the physiological range" is meant a pH in the range of approximately 7.2 to 8.0 inclusive, more typically in the range of approximately 7.2 to 7.6 inclusive.

"Recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide. "Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting procaryotic microorganisms or eucaryotic cell lines cultured as unicellular entities, are used interchangeably, and refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement to the original parent, due to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the

parent to be characterized by the relevant property, such as the presence of a nucleotide sequence encoding a desired peptide, are included in the progeny intended by this definition, and are covered by the above terms.

As used herein, "treatment" refers to any of (i) the prevention of infection or reinfection, as in a traditional vaccine, (ii) the reduction or elimination of symptoms, and (iii) the substantial or complete elimination of the pathogen in question. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

B. General Methods

Central to the present invention is the discovery that VLPs can serve as adjuvants or coadjuvants to enhance humoral and/or cell-mediated immune responses in a vertebrate subject when the VLPs are administered with a selected antigen. Surprisingly, the antigen need not be entrapped within the VLP in order for enhanced immunogenicity to result. Thus, the present invention does not require the use of linking agents, harsh chemical treatments, and the like, since the antigen of interest is not encapsulated or chemically conjugated to the VLP. Additionally, antigen size is not limited since the system does not depend on encapsulation of the antigen. Accordingly, the present system is useful with a wide variety of antigens and provides a powerful tool to prevent and/or treat a large number of infections.

VLPs for use as adjuvants can be formed from any viral protein, particle-forming polypeptide derived from the viral protein, or combination of viral proteins or fragments thereof, that have the capability of forming particles under appropriate

conditions. The requirements for the particle-forming viral protein are that if the particle is formed in the cytoplasm of the host cell, the protein must be sufficiently stable in the host cell in which it is expressed such that formation of virus-like structures will result, and that the polypeptide will automatically assemble into a virus-like structure in the cell of the recombinant expression system used. If the protein is secreted into culture media, conditions can be adjusted such that VLPs will form. Furthermore, the particle-forming protein should not be cytotoxic in the expression host and should not be able to replicate in the host in which the VLP will be used.

For example, it is known that some proteins can spontaneously form VLPs when pH is brought to an appropriate level. Similarly, some proteins require sufficient amounts of protein to be present in order for VLPs to form.

Methods and suitable conditions for forming particles from a wide variety of viral proteins are known in the art. For example, proteins derived from the hepatitis B virus (HBV) surface, such as the surface antigen, sAg, as well as the presurface sequences, pre-S1 and pre-S2 (formerly called pre-S), and any combination of these sequences, can spontaneously form particles upon expression in a suitable host cell, such as upon expression in mammalian, insect, yeast or Xenopus cells. Thus, HBV VLPs for use in the present invention can include particle-forming polypeptides of sAg, pre-S1 or pre-S2, as well as particle-forming polypeptides from any combination of the above, such as sAg/pre-S1, sAg/pre-S2, sAg/pre-S1/pre-S2, and pre-S1/pre-S2. See, e.g., "HBV Vaccines - from the laboratory to license: a case study" in Mackett, M. and Williamson, J.D., *Human*

Vaccines and Vaccination, pp. 159-176, for a discussion of HBV structure; and U.S. Patent Nos. 4,722,840, 5,098,704, 5,324,513, Beames et al., *J. Virol.* (1995) 69:6833-6838, Birnbaum et al., *J. Virol.* (1990) 64:3319-3330, Zhou et al., *J. Virol.* (1991) 65:5457-5464, for descriptions of the recombinant production of various HBV particles.

Additionally, particle-forming polypeptides derived from papillomavirus structural proteins L1 and L2, either alone or in combination, will find use herein as VLP adjuvants and coadjuvants. L1 and L2 proteins for use with the present invention can be derived from any of the various human and other animal papillomaviruses and subgroups thereof, including but not limited to, HPV-1, HPV-2, HPV-5, HPV-6, HPV-8, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33 and HPV-45. The sequences for L1 and L2 are known for various HPV types. See, e.g., *Human Papillomaviruses: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences* (Myers, G. et al., eds.) Los Alamos National Laboratory, Los Alamos, New Mexico, 1994-1996, for the sequences of various HPV types.

Methods for forming VLPs from L1 and L2, either alone or in combination, are known and reported in, e.g., U.S. Patent No. 5,437,951 (production of L1 VLPs in insect cells); WO 95/20659 (production of L2 VLPs in eucaryotic cells using vaccinia virus vectors); WO 93/02184 (production of L1/L2 VLPs in eucaryotic cells using vaccinia virus vectors); Kirnbauer et al. *J. Virol.* (1993) 67:6929-6936 (production of L1 and L1/L2 VLPs in insect cells); Heino et al., *Virology* (1995) 214:349-359 (production of L1 and L1/L2 VLPs in mammalian cells); Sasagawa et al., *Virology* (1995) 206:126-135 (production of L1 and L1/L2 VLPs in yeast cells); Zhou et al., *Virology* (1991) 185:251-257 (production of L1/L2 VLPs in

epithelial cells infected with vaccinia virus vectors).

Similarly, some rotavirus proteins are known to form VLPs upon expression. For example, rotavirus VP6 VLPs can be produced as described in U.S. Patent No. 5,503,833. Generally, VP6 spherical particles spontaneously form when the pH of a lysate containing recombinantly produced VP6 is brought to about pH 4.0 or greater. Bovine rotavirus VP2 spontaneously forms VLPs upon expression in baculovirus systems. See, e.g., Labbe et al., *J. Virol.* (1991) 65:2946-2952.

The yeast retrotransposon, Ty, encodes a set of proteins that assembles into VLPs. See, e.g., Mellor et al., *Nature* (1985) 318:583-586; Garfinkel et al., *Cell* (1985) 42:507-517; Adams et al., *Cell* (1987) 49:111-119. Ty VLPs can be formed from the primary translation product of the Ty element, p1, as well as the combination of p1 with p3 (pre-Ty VLP), and particle-forming proteolytic products of p1 and p3, upon expression in yeast. See, e.g., Adams et al., *Nature* (1987) 329:68-70.

VLPs for use in the present invention can also be derived from viral proteins from human parvovirus B19, such as the viral proteins VP1 and/or VP2 expressed in insect cells (see, Brown et al., *Virology* (1994) 198:477-488) and insect cell-expressed VP1, VP2 and/or VP3 proteins from polyomavirus (Delos et al., Virology (1993) 194:393-398; Forstova et al., *Human Gene Therapy* (1995) 6:297-306). Retrovirus gag proteins, such as those derived from Rous sarcoma virus (RSV) and Moloney murine leukemia virus (MLV) will also find use herein, as will the HIV-1 group-specific core antigen, p55*gag*, and deletion mutants thereof, which spontaneously form VLPs when expressed in eucaryotic cells. See, e.g., Wagner et al., *Arch. Virol.* (1992) 127:117-137; Wagner et al., *Virology*

(1994) 200:162-175; Brand et al., *J. Virol. Meth.* (1995) 51:153-168 and Wagner et al., *Virology* (1996) 220:128-140.

As explained above, VLPs can spontaneously form when the particle-forming polypeptide of interest is recombinantly expressed in an appropriate host cell. Thus, the VLPs for use in the present invention are conveniently prepared using recombinant techniques, well known in the art. In this regard, genes encoding the particle-forming polypeptide in question can be isolated from DNA libraries or directly from cells and tissues containing the same, using known techniques. See, e.g., Sambrook et al., *supra*, for a description of techniques used to obtain and isolate DNA. The genes encoding the particle-forming polypeptides can also be produced synthetically, based on the known sequences. The nucleotide sequence can be designed with the appropriate codons for the particular amino acid sequence desired. In general, one will select preferred codons for the intended host in which the sequence will be expressed. The complete sequence is generally assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, *Nature* (1981) 292:756; Nambair et al., *Science* (1984) 223:1299; Jay et al., *J. Biol. Chem.* (1984) 259:6311.

Once coding sequences for the desired particle-forming polypeptides have been isolated or synthesized, they can be cloned into any suitable vector or replicon for expression. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. See, generally, Sambrook et al, *supra*. The vector is then used to transform an appropriate host cell. Suitable expression systems

include, but are not limited to, bacterial, mammalian, baculovirus/insect, vaccinia, Semliki Forest virus (SFV), mammalian, yeast and Xenopus expression systems, well known in the art. Particularly preferred expression systems are mammalian, vaccinia, insect and yeast systems.

For example, a number of mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guillerimondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with baculovirus expression vectors include, *inter alia*, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*. See, e.g., Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987).

Viral vectors can be used for the production of particles in eucaryotic cells, such as those derived from the pox family of viruses, including vaccinia virus and avian poxvirus. Additionally, a vaccinia based infection/transfection system, as described in Tomei et al., *J. Virol.* (1993) 67:4017-4026 and Selby et al., *J. Gen. Virol.* (1993) 74:1103-1113, will also find use with the present

invention. In this system, cells are first transfected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the DNA of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA which is then translated into protein by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation product(s).

Depending on the expression system and host selected, the VLPS are produced by growing host cells transformed by an expression vector under conditions whereby the particle-forming polypeptide is expressed and VLPs can be formed. The selection of the appropriate growth conditions is within the skill of the art. If the VLPs are formed intracellularly, the cells are then disrupted, using chemical, physical or mechanical means, which lyse the cells yet keep the VLPs substantially intact. Such methods are known to those of skill in the art and are described in, e.g., *Protein Purification Applications: A Practical Approach*, (E.L.V. Harris and S. Angal, Eds., 1990)

The particles are then isolated using methods that preserve the integrity thereof, such as by gradient centrifugation, e.g., cesium chloride (CsCl) and sucrose gradients, and the like (see, e.g., Kirnbauer et al. *J. Virol.* (1993) 67:6929-6936), as well as standard purification techniques including, e.g., ion exchange and gel filtration chromatography.

Once obtained, the VLP adjuvants of the present invention can be incorporated into vaccine

compositions containing the desired antigen, or can be administered separately, either simultaneously with, just prior to, or subsequent to, an antigen-containing composition. The vaccine compositions can be used both for treatment and/or prevention of infection. Furthermore, the adjuvant formulations of the invention may be used to enhance the activity of antigens produced *in vivo*, i.e., in conjunction with DNA immunization.

The VLP adjuvants can be used in compositions for immunizing a vertebrate subject against one or more selected pathogens or against subunit antigens derived therefrom, or for priming an immune response to one or several antigens. Antigens that can be administered with the VLP adjuvants include proteins, polypeptides, antigenic protein fragments, oligosaccharides, polysaccharides, and the like. Similarly, an oligonucleotide or polynucleotide, encoding a desired antigen, can be administered with the VLP adjuvants for *in vivo* expression.

Antigens can be derived from a wide variety of viruses, bacteria, fungi, plants, protozoans and other parasites. For example, the present invention will find use for stimulating an immune response against a wide variety of proteins from the herpesvirus family, including proteins derived from herpes simplex virus (HSV) types 1 and 2, such as HSV-1 and HSV-2 gB, gD, gH, VP16 and VP22; antigens derived from varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) including CMV gB and gH; and antigens derived from other human herpesviruses such as HHV6 and HHV7. (See, e.g. Chee et al., *Cytomegaloviruses* (J.K. McDougall, ed., Springer-Verlag 1990) pp. 125-169, for a review of the protein coding content of cytomegalovirus; McGeoch et

al., *J. Gen. Virol.* (1988) 69:1531-1574, for a discussion of the various HSV-1 encoded proteins; U.S. Patent No. 5,171,568 for a discussion of HSV-1 and HSV-2 gB and gD proteins and the genes encoding therefor; Baer et al., *Nature* (1984) 310:207-211, for the identification of protein coding sequences in an EBV genome; and Davison and Scott, *J. Gen. Virol.* (1986) 67:1759-1816, for a review of VZV.)

Additionally, immune responses to antigens from the hepatitis family of viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis virus (HDV), hepatitis E virus (HEV), and hepatitis G virus, can also be conveniently enhanced using the VLP adjuvants. By way of example, the HCV genome encodes several viral proteins, including E1 (also known as E) and E2 (also known as E2/NSI), which will find use with the present invention (see, Houghton et al. *Hepatology* (1991) 14:381-388, for a discussion of HCV proteins, including E1 and E2). The δ-antigen from HDV can also be used with the present VLP adjuvant system (see, e.g., U.S. Patent No. 5,389,528, for a description of the δ-antigen).

Similarly, influenza virus is another example of a virus for which the present invention will be particularly useful. Specifically, the envelope glycoproteins HA and NA of influenza A are of particular interest for generating an immune response. Numerous HA subtypes of influenza A have been identified (Kawaoka et al., *Virology* (1990) 179:759-767; Webster et al. "Antigenic variation among type A influenza viruses," p. 127-168. In: P. Palese and D.W. Kingsbury (ed.), *Genetics of influenza viruses.* Springer-Verlag, New York). Thus, the immune response to any of these antigens may be enhanced when they are administered with the subject VLP adjuvants.

Other antigens of particular interest to be used in combination with the VLP adjuvants include antigens and polypeptides derived therefrom from human papillomavirus (HPV), such as one or more of the various early proteins including E6 and E7, tick-borne encephalitis viruses, HIV-1 (also known as HTLV-III, LAV, ARV, hTLR, etc.), including but not limited to antigens from the isolates HIV_{IIIb}, HIV_{SF2}, HIV_{LAV}, HIV_{LAI}, HIV_{MN}) such as gp120, gp41, gp160, gag and pol (see, e.g., Myers et al. Los Alamos Database, Los Alamos National Laboratory, Los Alamos, New Mexico (1992); Myers et al., *Human Retroviruses and Aids, 1990*, Los Alamos, New Mexico: Los Alamos National Laboratory; and Modrow et al., *J. Virol.* (1987) 61:570-578, for a comparison of the envelope gene sequences of a variety of HIV isolates).

Proteins derived from other viruses will also find use in the claimed methods, such as without limitation, proteins from members of the families Picornaviridae (e.g., polioviruses, etc.); Caliciviridae; Togaviridae (e.g., rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae; Birnaviridae; Rhabodoviridae (e.g., rabies virus, etc.); Filoviridae; Paramyxoviridae (e.g., mumps virus, measles virus, respiratory syncytial virus, etc.); Orthomyxoviridae (e.g., influenza virus types A, B and C, etc.); Bunyaviridae; Arenaviridae; Retroviradae, e.g., HTLV-I; HTLV-II; HIV-1; HIV-2; simian immundeficiency virus (SIV) among others. See, e.g. Virology, 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991), for a description of these and other viruses.

Particularly preferred bacterial antigens are derived from organisms that cause diphtheria, tetanus, pertussis, meningitis, and other pathogenic

states, including, without limitation, antigens derived from *Corynebacterium diphtheriae*, *Clostridium tetani*, *Bordetella pertusis*, *Neisseria meningitidis*, including serotypes *Meningococcus* A, B, C, Y and WI35 (MenA, B, C, Y and WI35), *Haemophilus influenza* type B (Hib), and *Helicobacter pylori*. Examples of parasitic antigens include those derived from organisms causing malaria and Lyme disease.

Combinations of antigens derived from the organisms above can be conveniently used to elicit immunity to multiple pathogens in a single vaccine. For example, a particularly preferred combination is a combination of bacterial surface oligosaccharides derived from MenC and Hib, conjugated to a nontoxic mutant carrier derived from a bacterial toxin, such as a nontoxic mutant of diphtheria toxin known as CRM_{197}. This conjugate is useful for preventing bacterial meningitis and is described in International Publication No. WO 96/14086, published May 17, 1996.

Furthermore, the methods described herein provide means for treating a variety of malignant cancers. For example, the system of the present invention can be used to enhance both humoral and cell-mediated immune responses to particular proteins specific to a cancer in question, such as an activated oncogene, a fetal antigen, or an activation marker. Such tumor antigens include any of the various MAGEs (melanoma associated antigen E), including MAGE 1, 2, 3, 4, etc. (Boon, T. *Scientific American* (March 1993):82-89); any of the various tyrosinases; MART 1 (melanoma antigen recognized by T cells), mutant ras; mutant p53; p97 melanoma antigen; CEA (carcinoembryonic antigen), among others.

It is readily apparent that the subject invention can be used to mount an immune response to a

wide variety of antigens and hence to treat or prevent a large number of diseases.

As explained above, the VLP formulations may or may not contain one or more antigens of interest. For example, antigens can be administered separately from the VLP compositions at the same or at different sites. In any event, one or more selected antigens will be administered in a "therapeutically effective amount" such that an immune response can be generated in the individual to which it is administered. The exact amount necessary will vary depending on the subject being treated; the age and general condition of the subject to be treated; the capacity of the subject's immune system to synthesize antibodies and/or mount a cell-mediated immune response; the degree of protection desired; the severity of the condition being treated; the particular antigen selected and its mode of administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. Thus, a "therapeutically effective amount" will fall in a relatively broad range that can be determined through routine trials. In general, a "therapeutically effective" amount of antigen will be an amount on the order of about 0.1 μg to about 1000 μg, more preferably about 1 μg to about 100 μg.

Similarly, the VLP adjuvant will be present in an amount such that the antigen displays "enhanced immunogenicity," as defined above, as compared to administration of the antigen alone, without the VLP adjuvant. Amounts which are effective for eliciting an enhanced immune response can be readily determined by one of skill in the art.

The compositions may additionally contain one or more "pharmaceutically acceptable excipients or vehicles" such as water, saline, glycerol, ethanol,

etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, biological buffers, and the like, may be present in such vehicles. A biological buffer can be virtually any solution which is pharmacologically acceptable and which provides the adjuvant formulation with the desired pH, i.e., a pH in the physiological range. Examples of buffer solutions include saline, phosphate buffered saline, Tris buffered saline, Hank's buffered saline, growth media such as Eagle's Minimum Essential Medium ("MEM"), and the like.

The antigen is optionally associated with a carrier which is a molecule that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycollic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as additional immunostimulating agents. Furthermore, the antigen may be conjugated to a bacterial toxoid, such as toxoid from diphtheria, tetanus, cholera, etc.

Coadjuvants in addition to the VLPs of the present invention, may also be used to enhance the effectiveness of the pharmaceutical compositions. Such coadjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59

(International Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (6) mucosal adjuvants such as those derived from cholera toxin (CT), pertussis toxin (PT), *E. coli* heat labile toxin (LT), and mutants thereof (see, e.g., International Publication Nos. WO 95/17211, WO 93/13202, and WO 97/02348); and (7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum and MF59 are preferred.

Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acteyl-normuramyl-L-alanyl-D-isogluatme (nor-MDP), N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-

alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

Once formulated, the compositions of the invention can be administered parenterally, e.g., by injection. The compositions can be injected either subcutaneously, intraperitoneally, intravenously or intramuscularly. Other modes of administration include oral and pulmonary administration, suppositories, mucosal and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals, chosen to maintain and/or reinforce the immune response, for example at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the subject and be dependent on the judgment of the practitioner. Furthermore, if prevention of disease is desired, the vaccines are generally administered prior to primary infection with the pathogen of interest. If treatment is desired, e.g., the reduction of symptoms or recurrences, the vaccines are generally administered subsequent to primary infection.

C. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts,

temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Example 1

In order to evaluate the immunogenicity in infant baboons of vaccines containing Hepatitis B virus (HBV) VLPs and *Haemophilus influenzae* type b (Hib) and/or Hib/Meningococcal C (MenC) conjugates, the following study was done.

Materials and Methods

A. Vaccines

The Hib/MenC conjugate vaccine and Hib conjugate vaccines used in the study included oligosaccharides independently conjugated to the protein carrier CRM_{197}, and were produced as described in International Publication No. WO 96/14086, published May 17, 1996.

The VLPs used were recombinant Hepatitis B virus (HBV) Pre-S2/S antigens. To produce the VLPs, a Pre-S2/sAg gene was incorporated into an expression vector and used to transfect DG44 Chinese hamster ovary (CHO) cells which had also been transfected with the dhfr gene. Growth of the cells and expression of the HBV particles was performed essentially as described in Michel et al., *Bio/Technology* (June 1985) 3:561-566 and Patzer et al., *Bio/Technology* (July 1986) 4:630-636.

The control vaccine formulation included a yeast-derived HBV S antigen adsorbed to alum.

On the day of vaccination, the glycoconjugate vaccines and the HBV VLP formulations were reconstituted together to provide a dose of approximately 5 μg of each of the saccharides, a total of 20 μg of CRM_{197} protein, and 2.5 μg of HBV VLPs.

B. Vaccine Groups

Infant baboons, 1.5 to 4 months of age at study initiation, were assigned to groups of 5 animals each, as shown in Table 1.

Table 1
Vaccine Groups

Group*	HBV	Hib	MenC	Adjuvant
1	Control Vaccine 2.5 μg	---	---	Alum
2	HBV VLPs	---	---	MF59
3	HBV VLPs	5 μg	---	MF59
4	HBV VLPs	5 μg	5 μg	MF59
5	---	5 μg	5 μg	MF59

*N=6 animals per group (1.5 to 4 mos. of age at study initiation)

Blood samples were collected prior to each immunization and 2 weeks after the third immunization.

Results

Figures 1 and 2 show the serum antibody responses of the infant baboons to HBV VLPs as determined by a commercial EIA (Abbott). The results are presented as the geometric mean of the antibody concentrations in IU/ml, ±95% Cl, on a log scale, for the four groups that were given vaccines containing HBV VLPs.

As can be seen, HBV VLPs alone, given with MF59, were highly immunogenic, achieving geometric mean titers of greater than 100 IU/ml after a single injection, greater than 1000 IU/ml after a second injection, and greater than 100,000 IU/ml after a third injection.

In contrast, the control vaccine given with alum was much less immunogenic, with no response to 1 injection, and 10-fold lower antibody responses to the second and third injections, compared to the experimental vaccines. Although not shown, there were no detectable hepatitis antibody responses in the fifth group which was given the MenC/Hib conjugate vaccines without VLPs. These results parallel the differences in immunogenicity between these two vaccines observed in clinical trials in healthy adults.

The presence of Hib, or Hib/MenC conjugate, decreased the hepatitis antibody responses, compared to those of the group receiving the Hepatitis B/MF59 alone.

This was true particularly after the first or second injections. However, after the third injection of the MF59-adjuvanted combination vaccines, the respective antibody responses to HBV were still 10-fold higher than those of the animals vaccinated with the control vaccine (geometric means 49,000 and 38,000 IU/ml versus 5000 IU/ml for the control HBV) ($p \leq 0.01$).

Serum anticapsular antibody responses of the infant baboons to the Hib conjugate vaccine, were determined using a radioantigen binding assay. Figures 3 and 4 show the geometric mean antibody concentrations in μg/ml on a log scale for the three groups given vaccines containing Hib/MenC given with MF59, HBV/Hib and MF59, and HBV/Hib/MenC with MF59.

All three groups showed robust antibody responses to Hib. Interestingly, the presence of HBV VLPs appeared to augment the Hib antibody responses. For example, after the third injection, the geometric mean antibody concentration was 35 μg/ml in the group receiving Hib/MenC and HBV with MF59, versus 10.8 μg/ml for the group receiving Hib/MenC/MF59 without

HBV. The Hib response to HBV/Hib without MenC was even higher (100 μg/ml).

Serum antibody responses of the infant baboons to the MenC conjugate vaccine were also measured. Results are shown in Figures 5A and 5B. Figure 5A shows the IgG anticapsular antibody responses determined by ELISA. Figure 5B depicts complement-mediated bactericidal antibody responses. The antibody responses to the MF59-adjuvanted MenC combination vaccine containing HBV VLPs were equivalent or higher than those of animals given the Hib/MenC vaccine alone with MF59. This was true for both IgG binding antibody determined by ELISA, and antibody functional activity determined with the bactericidal assay.

Table 2 summarizes the geometric antibody responses after the third injection for all groups. As can be seen, the animals given the control vaccine adsorbed with alum, or the experimental HBV VLP preparation with MF59, responded only to HBV. Further, the magnitude of the response was >20-fold higher to the experimental MF59 adjuvanted vaccine.

The HBV antibody responses to the two combination vaccines containing Hib, or Hib and MenC were approximately 2-fold lower than those of animals given HBV/MF59 alone. Nevertheless, they were still 8- to 10-fold higher than those observed with the control vaccine. As already noted, the Hib antibody responses to the two combination vaccines containing HBV VLPs were higher than those of animals given Hib/MenC/MF59 without HBV VLPs (group 5).

With respect to MenC conjugate, there also was evidence that HBV VLPs may augment the IgG MenC antibody responses.

Table 2
Serum Antibody Responses

	Geo. Mean Antibody (Post-3)			
	HBV VLPs	Hib	MenC	
Group/Dose, μg	(mlU/ml)	(μg/ml)	IgG, U/ml	1/Cidal
Control Vaccine	5,400	0.05	0.1	ND
MF59 with:				
HBV VLPs	120,000	0.07	0.1	ND
VLP/Hib	49,600	128.0*	0.1	ND
VLP/Hib/MenC	37,600	35.2*	53.5†	1,550
Hib/MenC	4	10.8*	26.5†	1,079

*P=0.07; †P≤0.03

Example 2

An experiment was also done in rabbits in order to evaluate the immunogenicity of vaccines containing human papillomavirus 6b (HPV 6b) L1 VLPs and HPV E7 as follows.

Materials and Methods

A. Vaccines

The recombinant proteins used in the vaccines of this study were cloned from HPV 6b as described. See, Schwarz, *EMBO J.* (1983) 2:2341-2348. The virus-like particle (VLP) vaccine consisted of baculovirus-derived Late 1 (L1) protein that had self-assembled into particles, that were purified through ion exchange and gel filtration chromatography. The VLPs were approximately 50nm in size, and closely resembled HPV virions (Greer, *J. Clin. Micro.* (1995) 33:2058-2063). The second vaccine consisted of *E. coli*-derived Early 7 (E7) protein.

On the day of vaccination, the vaccines were prepared such that a dose of VLP vaccine would contain 17 μg of HPV 6 L1 VLPs, while the E7 vaccine dose would contain 50 μg, and the L1/E7 combined vaccine dose would contain 17 μg of VLP and 50 μg of E7. The total volume of all the vaccines was 0.5 ml of which 0.25 ml was MF59 coadjuvant.

B. Vaccine Groups

Young adult, female New Zealand white rabbits were assigned to one of three possible groups (20 animals per group) as shown in Table 3. The animals received vaccines containing either VLP, E7, or a combination of L1 and E7.

Table 3
Vaccine Groups

Group	Number of Animals	L1 Antigen	E7 Antigen	Co-adjuvant	Total Vaccine Volume	Site of Injec-tion
L1	20	17 μg	---	MF59	0.5 ml	Hind leg
L1/E7	20	17 μg	50 μg	MF59	0.5 ml	Hind leg
E7	20	---	50 μg	MF59	0.5 ml	Hind leg

C. Immunization and Bleeding Schedules

The rabbits were given intramuscular (hind leg) vaccinations during week 0, 3, and 6. Blood samples were collected during weeks 0, 4, 5, 7 and 8.

D. Determination of Antigen-Specific Antibody Response

Antibody responses were measured using antigen-specific ELISAs. Titers were determined for each serum at the dilution that resulted in an optical density of 0.5.

Results

The rabbit serum titer results for E7 and L1 antigens are summarized in Figure 6. The results presented on a log scale, are the arithmetic mean of the antibody titers for the groups. The antibody titers generated from the E7 alone with MF59 vaccine were significantly lower than the VLPs after the second and third vaccinations (313 versus 106,000; $p<0.0001$; and 1,400 versus 65,000; $p<0.0001$). However, anti-E7 mean titers generated by the E7/VLP combination appeared augmented in the animals injected with combined E7/L1 vaccine (and MF59) as compared to E7 alone with MF59 (825 versus 313; 1,567 versus 1,363). In addition, the median titer of E7/L1 was significantly higher at weeks 5 and 8 at the 5% level (*p*-values 0.0018 and 0.0125, respectively). Furthermore, the number of non-responders was statistically significantly lower for E7/L1 than for E7 (*p*-value 0.023 Fisher's exact test).

The data indicates that the VLPs alone with MF59 were highly immunogenic, resulting in titers of 482 after the first injection and 106,000 and 65,000 after the second and third injections. However, anti-VLP mean titers for the combined VLP/E7 vaccine group after the second immunization were reduced (106,000 versus 89,000) and reduced significantly after the 3 immunization (65,000 versus 35,000; $p=0.001$).

Thus, novel VLP adjuvant compositions and methods for using and making the same are disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

We claim:

1. A vaccine composition comprising a virus-like particle (VLP) adjuvant, a selected antigen and a pharmaceutically acceptable excipient, wherein said selected antigen is distinct from said VLP.

2. The vaccine composition of claim 1, further comprising a coadjuvant.

3. The vaccine composition of claim 2, wherein said coadjuvant comprises MF59.

4. The vaccine composition of any of claims 1-3, wherein said VLP is formed from one or more particle-forming polypeptides of a hepatitis surface antigen.

5. The vaccine composition of claim 4, wherein said particle-forming polypeptide is at least one hepatitis surface antigen selected from the group consisting of sAg, pre-S1 and pre-S2.

6. The vaccine composition of claim 5, wherein said VLP is formed from sAg and pre-S2.

7. The vaccine composition of any of claims 1-3, wherein said VLP is formed from a particle forming polypeptide of papillomavirus L1 and/or L2.

8. The vaccine composition of any of claims 1-7, wherein said selected antigen is a MenC/Hib oligosaccharide conjugate.

9. The vaccine composition of any of claims 1-7, wherein said selected antigen is an HPV E7.

10. Use of a virus-like particle (VLP) adjuvant and a selected antigen distinct from said VLP, for the manufacture of a composition useful for producing an enhanced immune response in a vertebrate subject.

11. A use according to claim 10, wherein said VLP is formed from one or more particle-forming polypeptides of a hepatitis surface antigen.

12. A use according to claim 11, wherein said particle-forming polypeptide is at least one hepatitis surface antigen selected from the group consisting of sAg, pre-S1 and pre-S2.

13. A use according to claim 12, wherein said VLP is formed from sAg and pre-S2.

14. A use according to claim 10, wherein said VLP is formed from a particle forming polypeptide of papillomavirus L1 and/or L2.

15. A use according to any of claims 10-14, wherein said selected antigen is a MenC/Hib oligosaccharide conjugate.

16. A use according to any of claims 10-14, wherein said selected antigen is HPV E7.

17. A method for preparing an adjuvant formulation comprising providing a virus-like particle (VLP) and combining the VLP with a pharmaceutically acceptable excipient.

18. The method of claim 17 further comprising combining a selected antigen with said VLP and said pharmaceutically acceptable excipient.

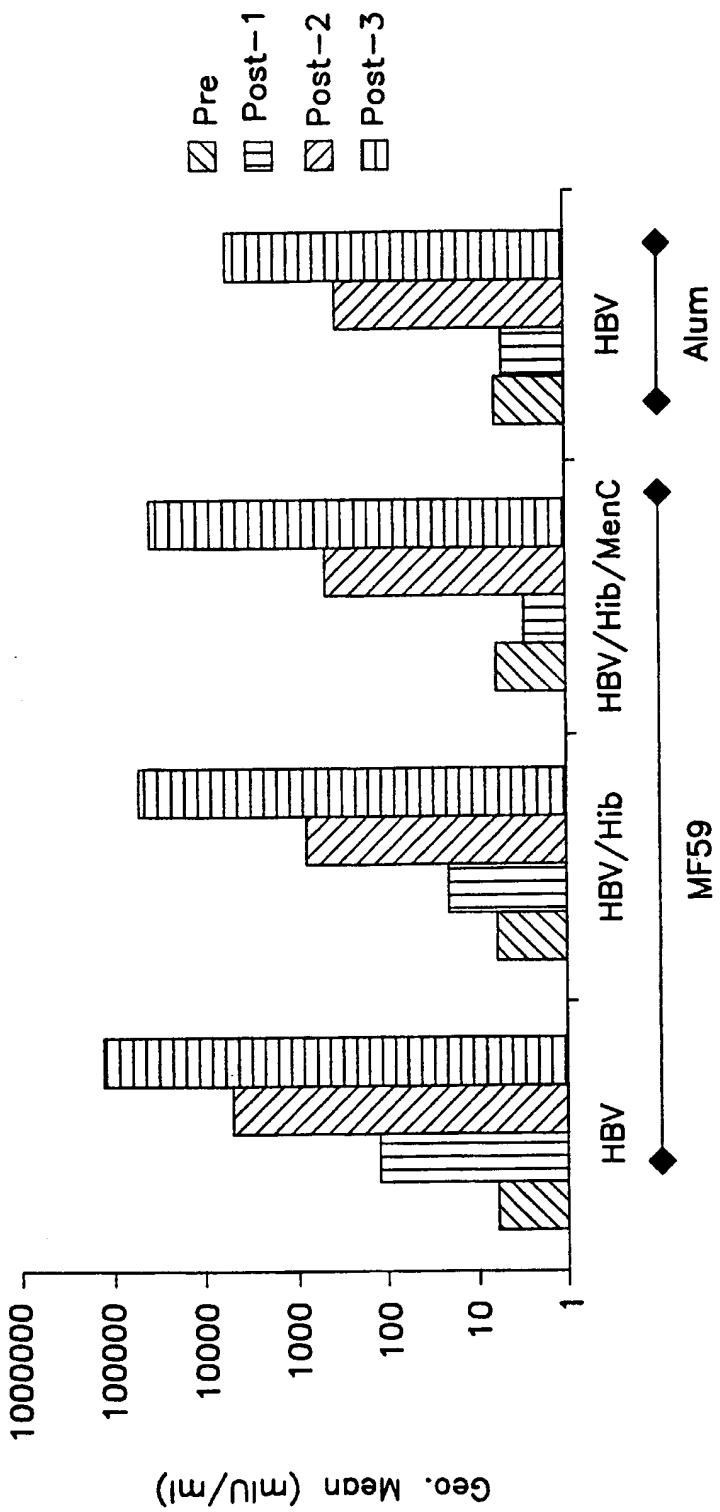
Pre
Post-1
Post-2
Post-3
Geo. Mean (mIU/ml)
1000000
100000
10000
1000
100
10
1
HBV
HBV/Hib
HBV/Hib/MenC
HBV
MF59
Alum

FIG. 1

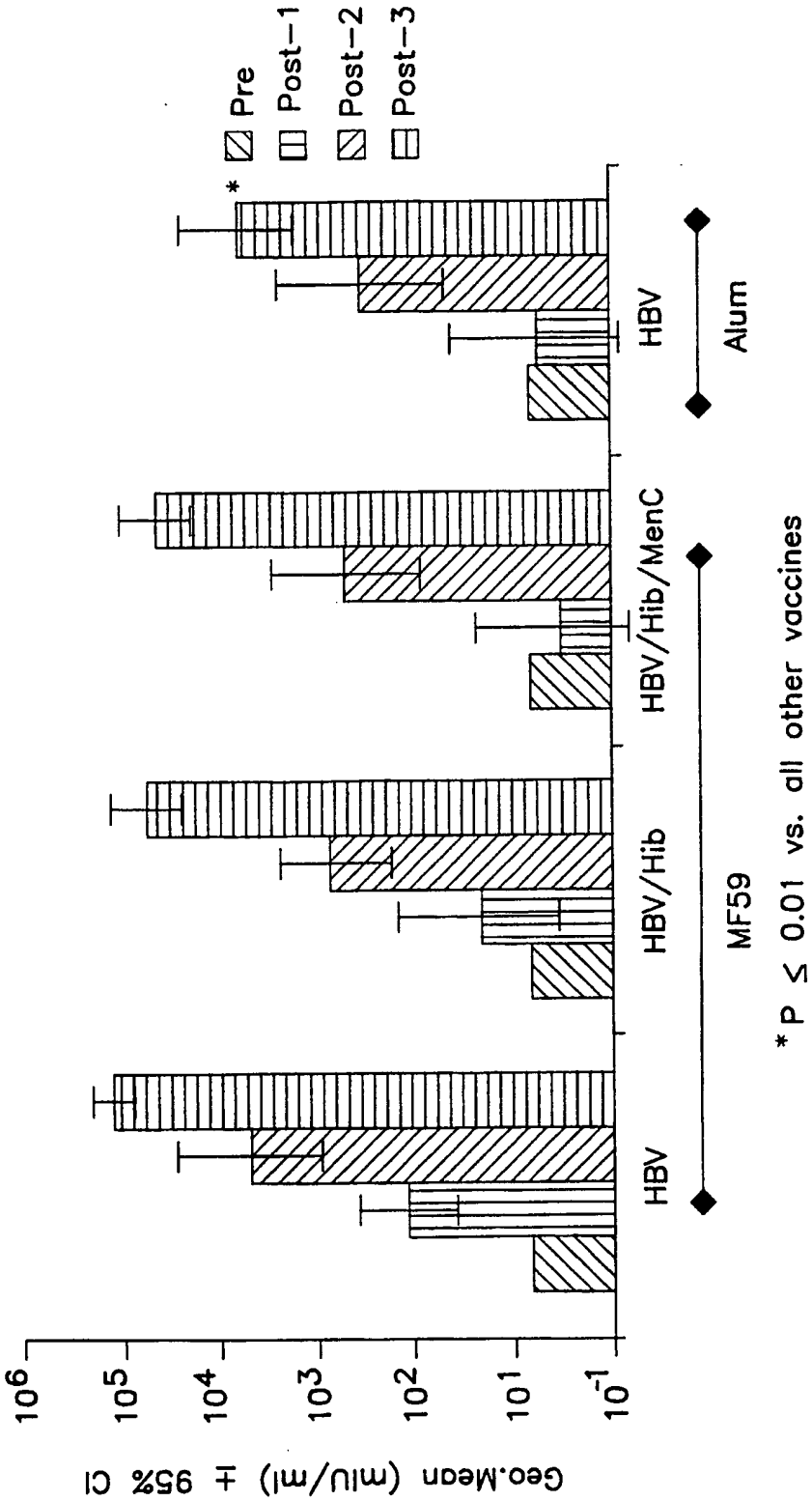
Pre
Post-1
Post-2
Post-3
*
HBV
Alum
HBV/Hib/MenC
HBV/Hib
HBV
MF59
* P ≤ 0.01 vs. all other vaccines
Geo.Mean (mIU/ml) ± 95% CI
10^6
10^5
10^4
10^3
10^2
10^1
10^-1

FIG. 2

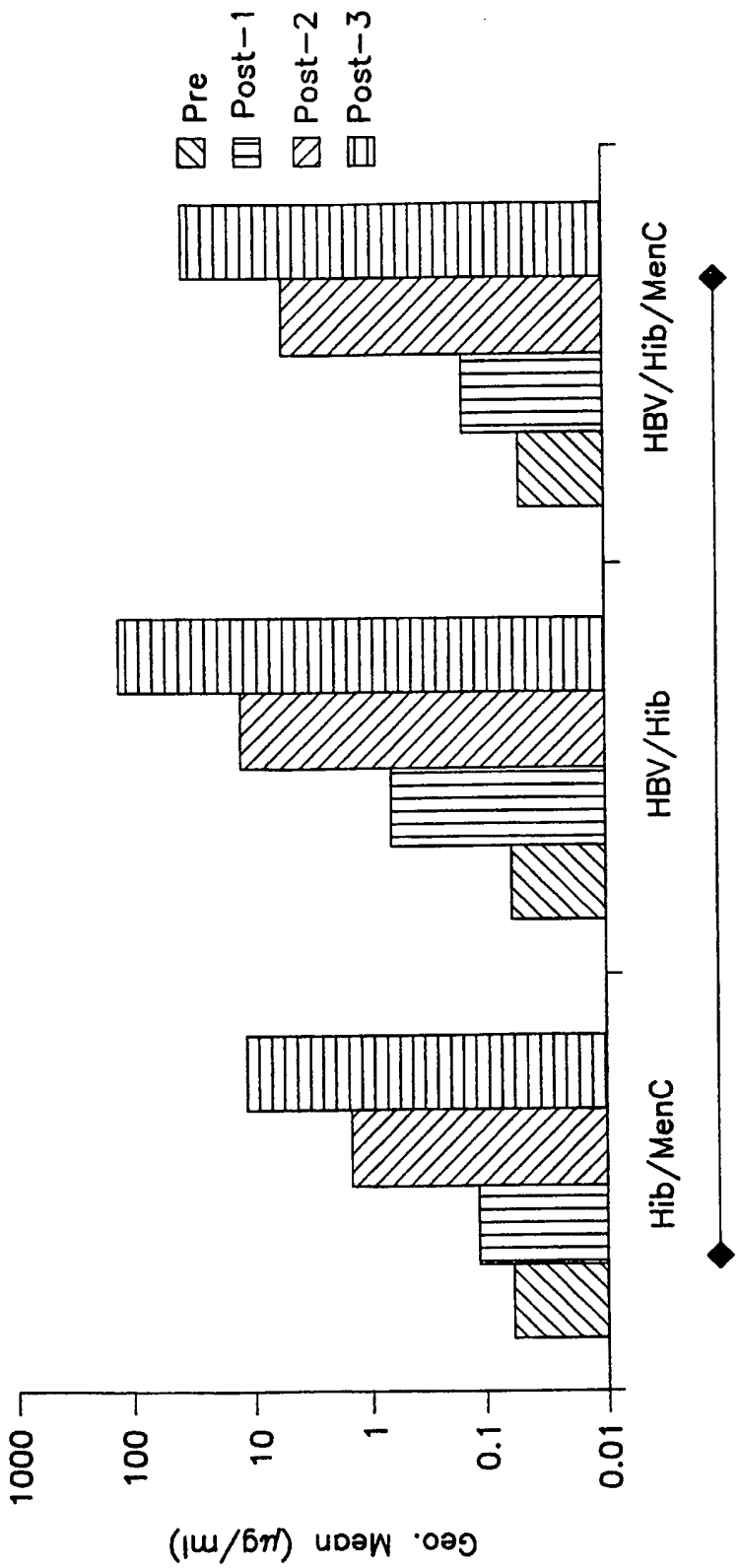
Pre
Post-1
Post-2
Post-3
HBV/Hib/MenC
HBV/Hib
Hib/MenC
MF59
1000
100
10
1
0.1
0.01
Geo. Mean (μg/ml)

FIG. 3

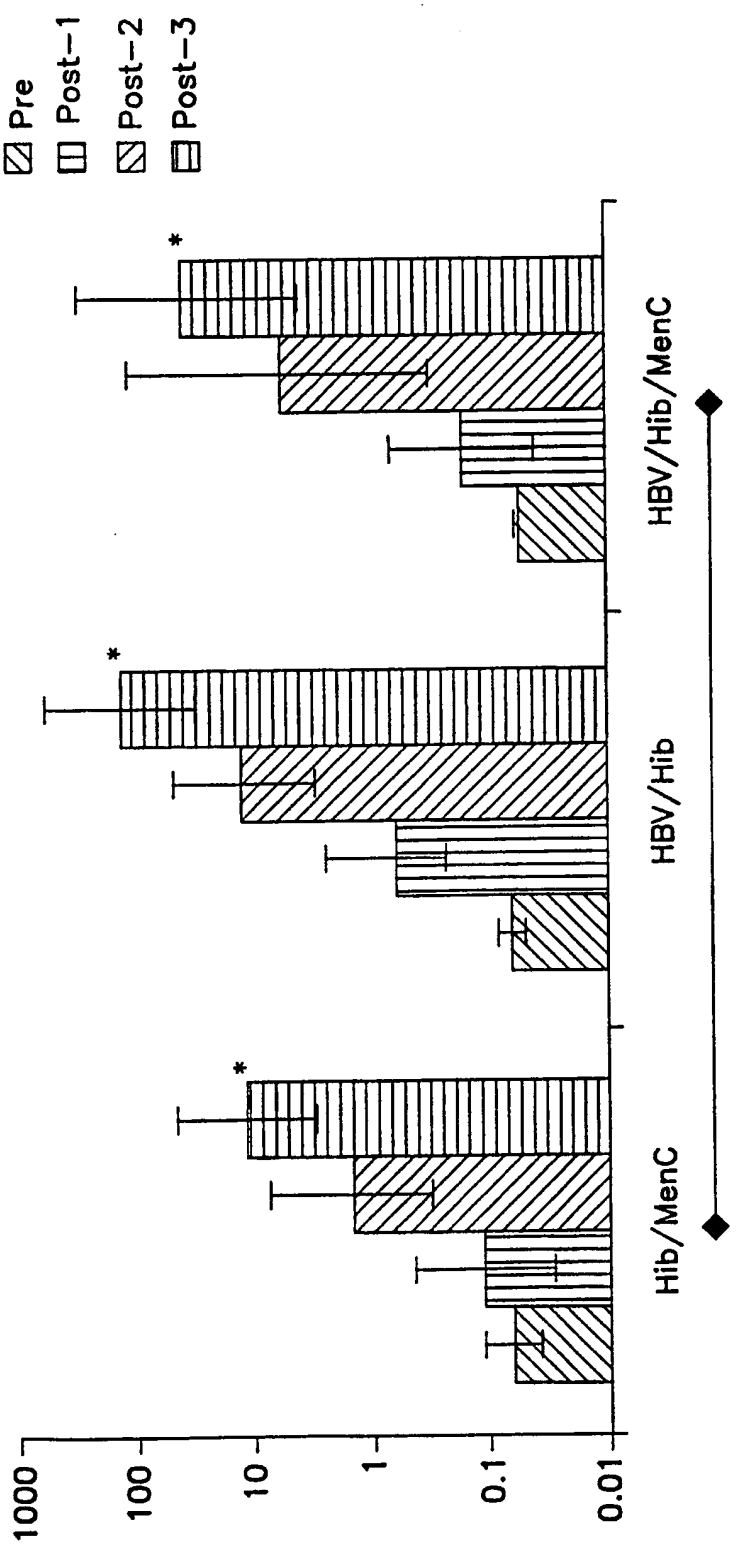
Pre
Post-1
Post-2
Post-3
Geo. Mean (μg/ml) ± 95% CI
1000
100
10
1
0.1
0.01
*
*
*
HBV/Hib/MenC
HBV/Hib
Hib/MenC
MF59
*P=0.07

FIG. 4

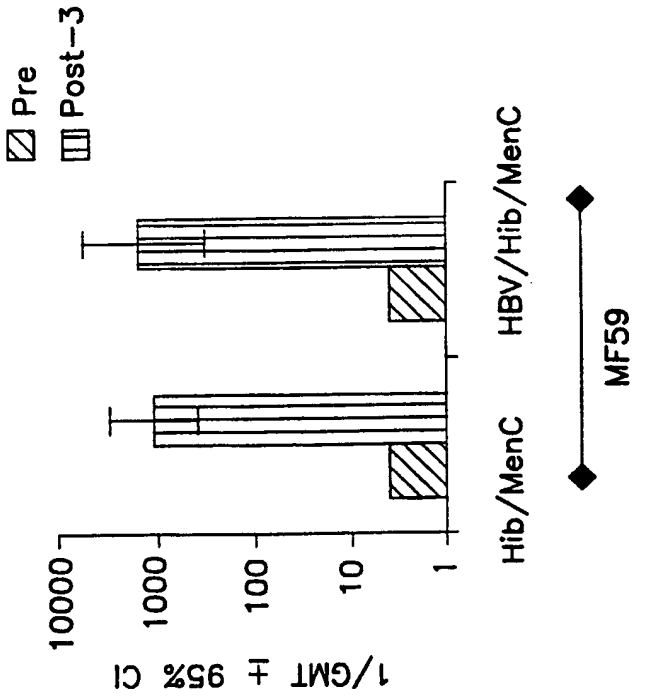

FIG. 5B

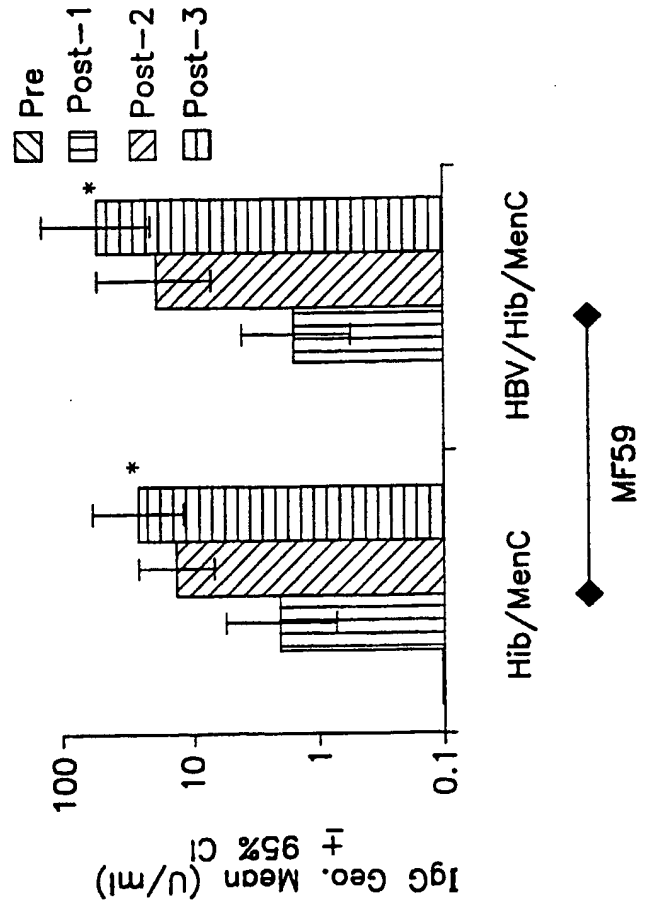

FIG. 5A

*P ≤ 0.03

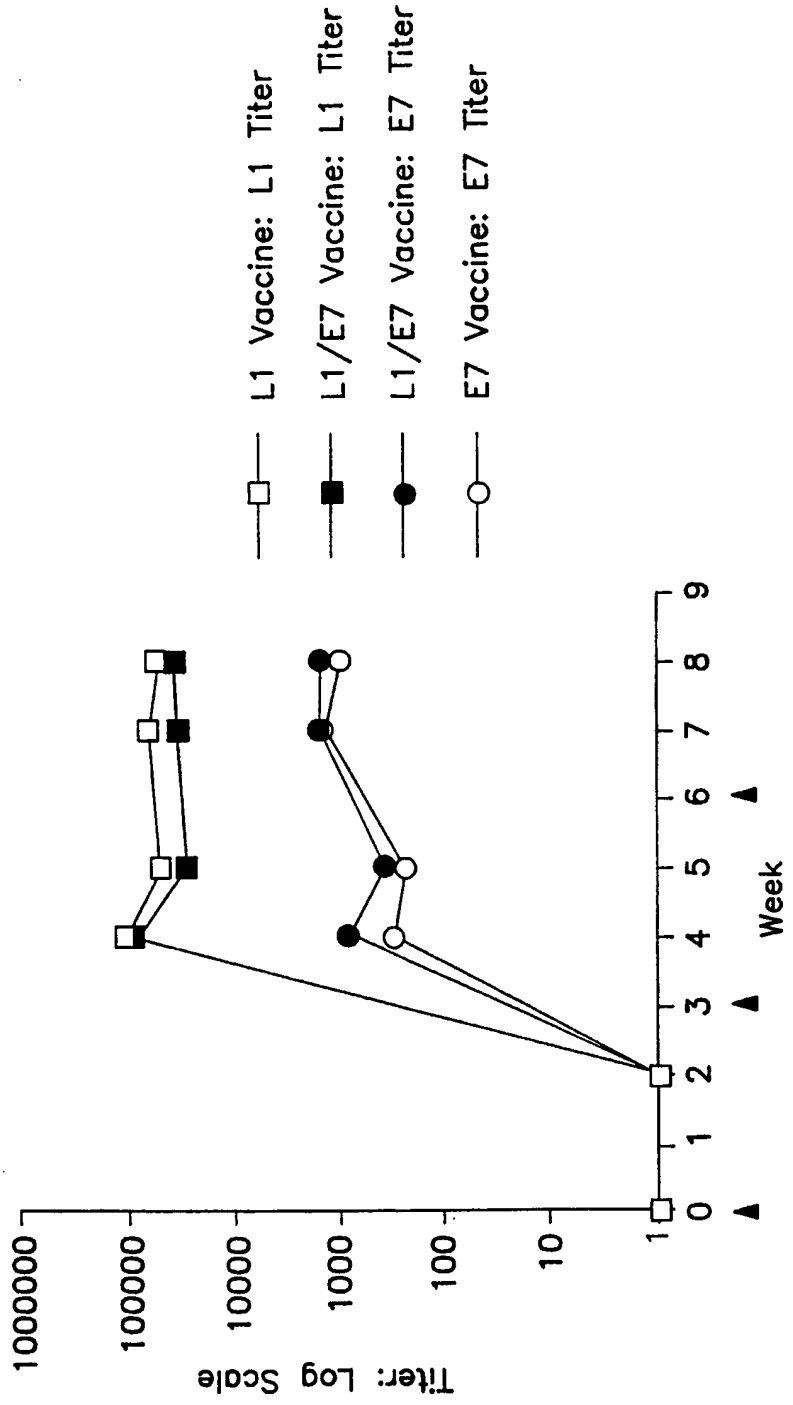
L1 Vaccine: L1 Titer
L1/E7 Vaccine: L1 Titer
L1/E7 Vaccine: E7 Titer
E7 Vaccine: E7 Titer
Titer: Log Scale
1000000
100000
10000
1000
100
10
1
0 1 2 3 4 5 6 7 8 9
Week

FIG. 6

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 98/08146

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39 A61K39/29 A61K39/102 A61K39/095

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 19267 A (SCHWEIZ SERUM & IMPFINST) 12 November 1992 see page 6 - page 7 see page 8, paragraph 2 see page 9, paragraph 2 see page 10, paragraph 2 ---	1,10,17, 18
X	WO 93 24148 A (SMITHKLINE BEECHAM BIOLOG) 9 December 1993 see page 6, line 7 - line 27; examples 1-3 ---	1,2,4-6, 9-13
X	WO 96 14086 A (BIOCINE SPA ;CECCARINI COSTANTE (IT); COSTANTINO PAOLO (IT); ASCEN) 17 May 1996 see page 6, line 18 - line 32; claims 1-9 --- -/--	1,8,10, 15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 August 1998	28/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fernandez y Branas,F

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INTERNATIONAL SEARCH REPORT

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PCT/US 98/08146

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 722 840 A (VALENZUELA PABLO D T ET AL) 2 February 1988 cited in the application see the whole document	1,2,4-6, 10-12
X	EP 0 259 149 A (UNIV SASKATCHEWAN) 9 March 1988 see the whole document	1,2,10
X	TINDLE R.W. ET AL: "Chimeric hepatitis B core antigen particles containing B- and Th- epitopes of human papillomavirus type 16 E7 protein induce specific antibody and T-helper responses in immunised mice" VIROLOGY, vol. 200, 1994, pages 547-557, XP002074501 see the whole document	1,2, 9-11,16
A	US 5 503 833 A (REDMOND MARK J ET AL) 2 April 1996 cited in the application see the whole document	1-18
A	US 5 618 536 A (LOWY DOUGLAS R ET AL) 8 April 1997 see the whole document	1-18
A	WO 93 02184 A (UNIV QUEENSLAND ;CLS LTD (AU)) 4 February 1993 see the whole document	1-18
A	M.F.POWELL AND M.J. NEWMAN: "vaccine design. The subunit and adjuvant approach" 1996 , PLENUM PRESS , NEW YORK XP002074502 see page 183	3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

In. itional Application No
PCT/US 98/08146

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9219267 A	12-11-1992	AU 655823 B	12-01-1995
		AU 1745692 A	21-12-1992
		CA 2086831 A	09-11-1992
		EP 0538437 A	28-04-1993
		JP 6500128 T	06-01-1994
		US 5565203 A	15-10-1996
WO 9324148 A	09-12-1993	AP 567 A	25-11-1996
		AU 1648097 A	29-05-1997
		AU 4315693 A	30-12-1993
		CA 2136429 A	09-12-1993
		CN 1085450 A	20-04-1994
		CZ 9402892 A	13-09-1995
		EP 0642355 A	15-03-1995
		EP 0835663 A	15-04-1998
		FI 945483 A	20-01-1995
		HU 71791 A	28-02-1996
		JP 7508267 T	14-09-1995
		MX 9302982 A	01-12-1993
		NO 944475 A	18-01-1995
		NZ 253065 A	28-10-1996
		PL 174077 B	30-06-1998
		SI 9300271 A	31-12-1993
		SK 142194 A	09-08-1995
		ZA 9303541 A	21-06-1994
WO 9614086 A	17-05-1996	EP 0789587 A	20-08-1997
US 4722840 A	02-02-1988	CA 1263618 A	05-12-1989
		DE 3584866 A	23-01-1992
		EP 0175261 A	26-03-1986
		HK 140195 A	08-09-1995
		JP 2531498 B	04-09-1996
		JP 7039379 A	10-02-1995
		JP 2659084 B	30-09-1997
		JP 7046997 A	21-02-1995
		JP 2068036 C	10-07-1996
		JP 7108920 B	22-11-1995
		JP 61129135 A	17-06-1986

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ational Application No
PCT/US 98/08146

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0259149 A	09-03-1988	AU 608769 B	18-04-1991
		AU 7791887 A	10-03-1988
		CA 1319101 A	15-06-1993
		DE 3788475 D	27-01-1994
		DE 3788475 T	07-04-1994
		DK 171602 B	17-02-1997
		ES 2060603 T	01-12-1994
		IE 61417 B	02-11-1994
		JP 63218627 A	12-09-1988
		US 5374426 A	20-12-1994
		US 5071651 A	10-12-1991
US 5503833 A	02-04-1996	PT 100090 A	30-07-1993
		AT 141515 T	15-09-1996
		AU 660168 B	15-06-1995
		AU 1176892 A	07-09-1992
		CA 2101735 A	05-08-1992
		WO 9213568 A	20-08-1992
		DE 69213030 D	26-09-1996
		EP 0660723 A	05-07-1995
		JP 6506667 T	28-07-1994
US 5618536 A	08-04-1997	US 5437951 A	01-08-1995
		AU 3828495 A	02-05-1996
		EP 0789766 A	20-08-1997
		JP 10506796 T	07-07-1998
		WO 9611274 A	18-04-1996
		AU 683220 B	06-11-1997
		AU 4847593 A	29-03-1994
		CA 2143845 A	17-03-1994
		EP 0662132 A	12-07-1995
		JP 8504087 T	07-05-1996
		WO 9405792 A	17-03-1994
		US 5756284 A	26-05-1998
		US 5709996 A	20-01-1998
		US 5744142 A	28-04-1998
		US 5716620 A	10-02-1998
WO 9302184 A	04-02-1993	AU 651727 B	28-07-1994
		EP 0595935 A	11-05-1994

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int .tional Application No
PCT/US 98/08146

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9302184 A		JP 7505042 T	08-06-1995